

Collagen microstructure basis for the correlation between acoustic and biomechanical properties
of the cornea

Undergraduate Honors Thesis

Presented in Partial Fulfillment of the Requirements for
Graduation with Honors Research Distinction

By

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Abstract

Biomechanical properties of the tissue comprising the eye, including the cornea and sclera, are important for the function of the eye. Knowing the mechanical properties of the eye will help develop and improve vision technology and treatments. The noninvasive assessment of *in vivo* mechanical properties is a challenge in the fields of biomechanics and ophthalmology due to a current lack of available and accurate technology. Recent findings indicate that acoustic impedance and the Young's modulus of the cornea are strongly correlated. However, the mechanisms responsible for this correlation remain unclear. This may result from the microstructure, which includes the type and arrangement of collagen fibers. In this study, fresh canine corneas were dissected and acoustically and mechanically tested through amplitude-mode ultrasound and strain-controlled rheometry, respectively. The tissue samples were then prepared for the collagen assay following acoustic and mechanical testing. The collagen content was assessed through a hydroxyproline collagen assay. The preliminary results showed a potential trend for correlations between acoustic properties and collagen content, but the methods will require adjustments and further investigation.

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Chapter 1: Introduction

Biomechanical properties of the tissue comprising the eye, including the cornea and sclera, provide information regarding the function and condition of the eye. Through the cornea, clinically important parameters, such as the intraocular pressure (IOP), can be determined. Changes in the biomechanical properties of the cornea may affect IOP measurements and can result in inaccurate measurements [1, 2]. Ablative refractive surgery, which involves the controlled removal of corneal tissue, may lead to biomechanical changes in the cornea [3]. These changes are difficult to assess and characterize using current methods. A current method of assessing the *in vivo* properties of the eye, the Ocular Response Analyzer, is believed to lack the ability to measure mechanical properties independent of other physical factors in the eye, including the geometry of the cornea and the IOP [4, 5]. The assessment of *in vivo* mechanical properties is a challenge in the field.

A novel non-invasive ultrasound method has shown potential in addressing this challenge, based on the finding of a strong correlation between the acoustic impedance and the Young's modulus of the cornea [6]. However, it remains unclear what mechanisms are responsible for this correlation. Palko et al. has demonstrated that the sclera exhibits a correlation between the collagen solubility profile and mechanical properties, and this may be extended to the cornea [7]. As the macrostructural properties of the eye are likely related to the microstructure, it is hypothesized that the corneal collagen content influences both the mechanical and acoustic properties. This potentially related corneal collagen content can encompass total collagen content as well as the amount of crosslinked collagen. Collagen content can be assessed through a

hydroxyproline collagen assay, in which a collagen solubility profile can be developed. Some literature suggests that highly crosslinked collagen is insoluble in many conditions, such as a pepsin digest, and this correlates with the mechanical stiffness of the tissue [7, 8]. Understanding the microstructural mechanisms will lead to further insight and assist in the clinical application of this correlation, potentially allowing for the development of a noninvasive method of measuring *in vivo* corneal stiffness [9]. A noninvasive tool will help gauge the effectiveness of current procedures and techniques, such as ablative refractive surgery and corneal cross-linking [3, 10]. Quantifying the mechanical integrity of the eye can determine the viability of surgical intervention and also assist in early discovery of conditions, such as corneal ectasia, which involves thinning of the cornea.

This study will examine the collagen content, specifically pepsin-soluble and pepsin-insoluble content, following corneal acoustic and mechanical testing.

Objective

To develop methods to investigate the relationships between corneal collagen content, Young's modulus, and the acoustic impedance of canine cornea

Hypothesis

It is hypothesized that the corneal collagen content is positively correlated with the mechanical and acoustic properties of the cornea in a canine model and can provide an explanation regarding the mechanisms responsible for the relationship between acoustic and biomechanical properties of the cornea.

Chapter 2: Methodology

Fresh canine eyes (N=12) were obtained from a local animal shelter. Canines were a variety of breed and approximately 1-5 year(s) old based upon veterinarian technician estimates. All dogs were medium size; no large or small canine donors were used for this study, and therefore, no large or small eyes were used. Eyes were enucleated within 20 minutes following euthanization and were transported in vials in a moist and buffered environment.

Upon returning to the lab, cornea thickness was measured via a pachymeter. Each eye underwent the following order of analysis: pachymetry, acoustic measurements, mechanical testing, and collagen content analysis. A flow chart of the testing can be seen below in Figure 2-1. Each procedure is described in the following paragraphs.

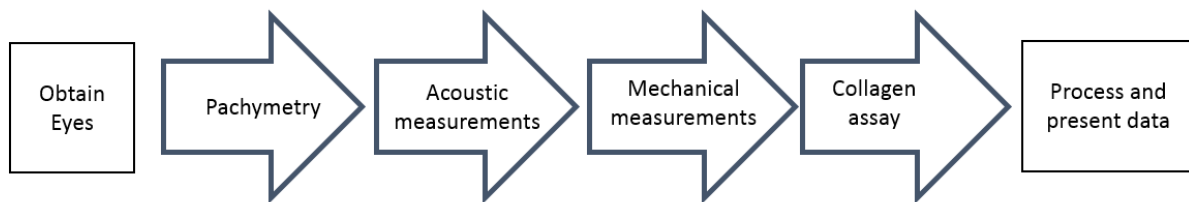


Figure 2-1: Flow chart of experimental methodology

Acoustic Testing

Amplitude mode (A-mode) ultrasound was used to measure the acoustic properties of the cornea, using the techniques reported by He and Liu [6]. The ultrasonic measurement system is composed of a transducer, a pulser-receiver, a digitizer, and a computer, as seen in Figure 2-2 below. Z_w refers to the acoustic impedance of water, and Z_s refers to the acoustic impedance of the sample. Sample globes were submerged in a saline bath during the ultrasonic measurements as ultrasound requires a medium to propagate. The acoustic echoes from the cornea were acquired and analyzed based on reflection amplitudes to obtain the acoustic impedance. An acrylic reference material was utilized in the calculation of acoustic impedance. Maximum peak-

to-peak wave values were obtained indicating that the transducer was nearly perpendicular to the sample. A sample wave can be viewed below in Figure 2-3 below. A_1 refers to the anterior reflection amplitude, and A_2 refers to the posterior reflection amplitude.

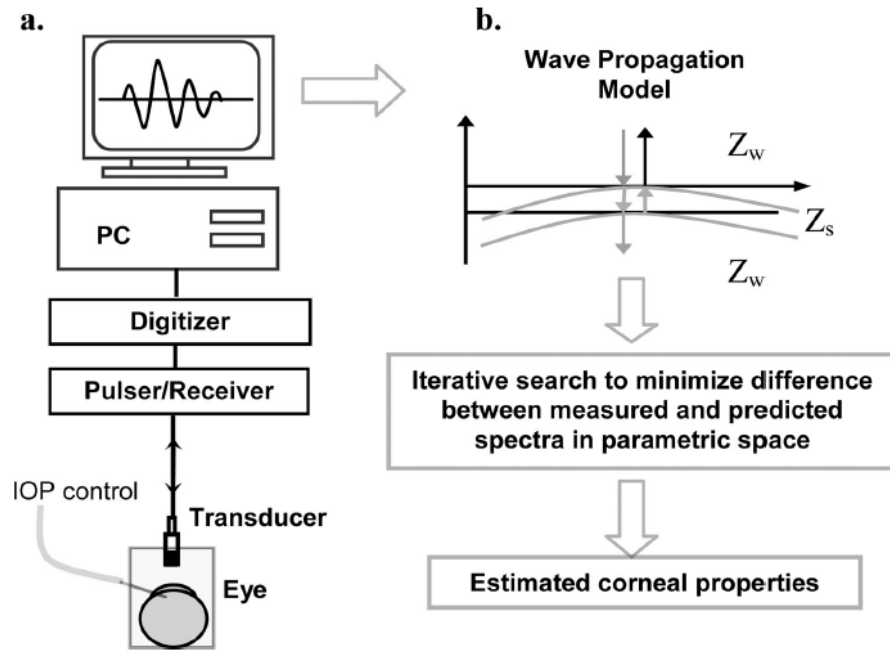


Figure 2-2: Ultrasonic measurement system and model [6]

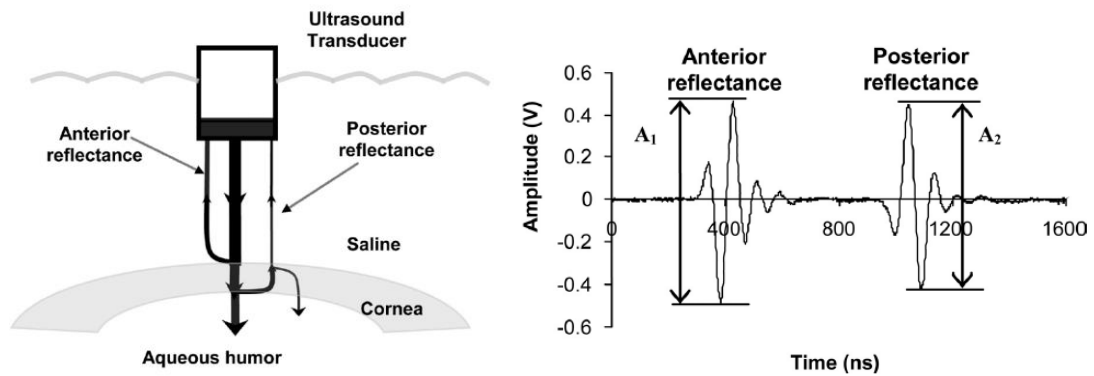


Figure 2-3: Ultrasound wave propagation and reflectance through the cornea [6]

Mechanical Testing

After acoustic testing, corneas were prepared for uniaxial tensile testing. Mechanical testing occurred within approximately 4 hours of enucleation. Corneas and approximately 1-2 mm of the surrounding sclera were dissected from the globes and placed in wells of transfer medium. Optisol (Chiron Ophthalmics, Irvine, California) solution was used as the transfer medium for preservation and to control cornea swelling. Corneas were cut into strips, one strip per eye, in the nasal-temporal direction immediately before mechanical testing using surgical blades. The nasal-temporal direction was easily identified by the vasculature of the globes. Strips were approximately 3-4 mm in width and typically had approximately 9-11 mm of corneal tissue in the nasal-temporal direction. Once strips were cut, Optisol solution was frequently applied, every 1-2 minutes, to the strips to maintain tissue hydration when samples were not in transfer wells. This protocol was adapted from the method reported by Tang et al and can be viewed in the Appendix as Tensile Testing (RSA) Protocol [11]. The sample thickness was previously measured through a pachymeter before acoustic testing, and sample width was measured via calipers after being cut into strips. Sample dimensions were entered into a computer program that used a rheometer system to calculate stress and strain from controlled testing. A sketch of the tensile testing setup can be viewed below in Figure 2-4. Samples were clamped at the sclera such that only corneal tissue was between the upper and lower clamp. Preloading of 5.0 g was applied to prepare the samples for consistent deformation. The samples were deformed up to a strain of 6%. Force, or indirectly stress, was recorded to calculate Young's secant modulus. After mechanical testing, samples were frozen for future collagen assays.

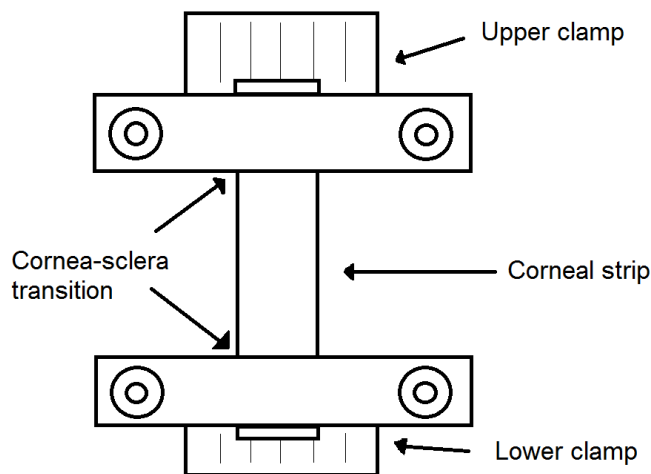


Figure 2-4: Corneal strip mechanical testing apparatus

Collagen Analysis

A hydroxyproline assay was performed to quantify the amounts of pepsin-soluble and pepsin-insoluble collagen in the cornea samples. The protocol was adapted from an assay designed to determine the collagen solubility profile for sclera and tendon [12, 13]. A detailed protocol can be viewed in the Appendix. The collagen solubility profile for this study includes pepsin-soluble and pepsin-insoluble collagen. Samples were removed from the freezer and were lyophilized to dry the tissue and obtain dry weight. Samples were then rehydrated in distilled water, cut into smaller pieces with surgical scissors, and then mechanically homogenized using a Fisher Scientific Tissuemiser Homogenizer (Fisher Scientific, Waltham, Massachusetts) to maximize tissue surface area. Samples underwent a pepsin digest in a vortex machine over 24 hours at 4°C. Samples were then centrifuged at 15,000 rpm, and the supernatant solution (pepsin-soluble collagen) was transferred to another tube and stored at 4°C. The remaining pepsin-insoluble collagen was fully mechanically homogenized. Samples were hydrolyzed in alkali in an autoclave for 30 minutes and oxidized with chloramine-T at room temperature for 25 minutes.

Ehrlick's reagent was added to samples, and they were placed in an oven at 65°C for 20 minutes to produce a chromophore. Figure 2-5 below is a sketch of all the reagents in a test tube.

Absorbance of the chromophore was measured at 555nm to determine collagen content against a curve of known concentrations. It is assumed that 14% of collagen is hydroxyproline.

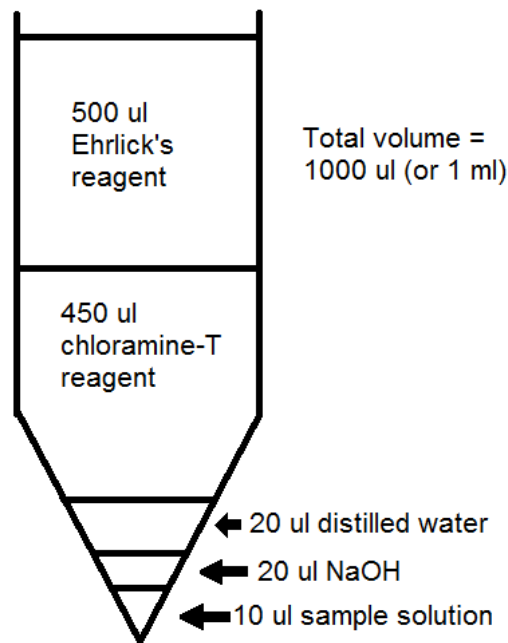


Figure 2-5: Volume of reagents for collagen assay

Data Processing and Calculations

Acoustic testing data was processed in MATLAB. A MATLAB script file that processed the data files was developed by Dr. Hong Chen. The code would locate and plot the anterior and posterior reflections of the cornea, plot the incident wave form spectrum, and calculate the acoustic impedance of the cornea. The program requires the input of gain/attenuation of the wave forms produced from the eye and reference material. A sample output of the program can be viewed in Figure 2-6 below.

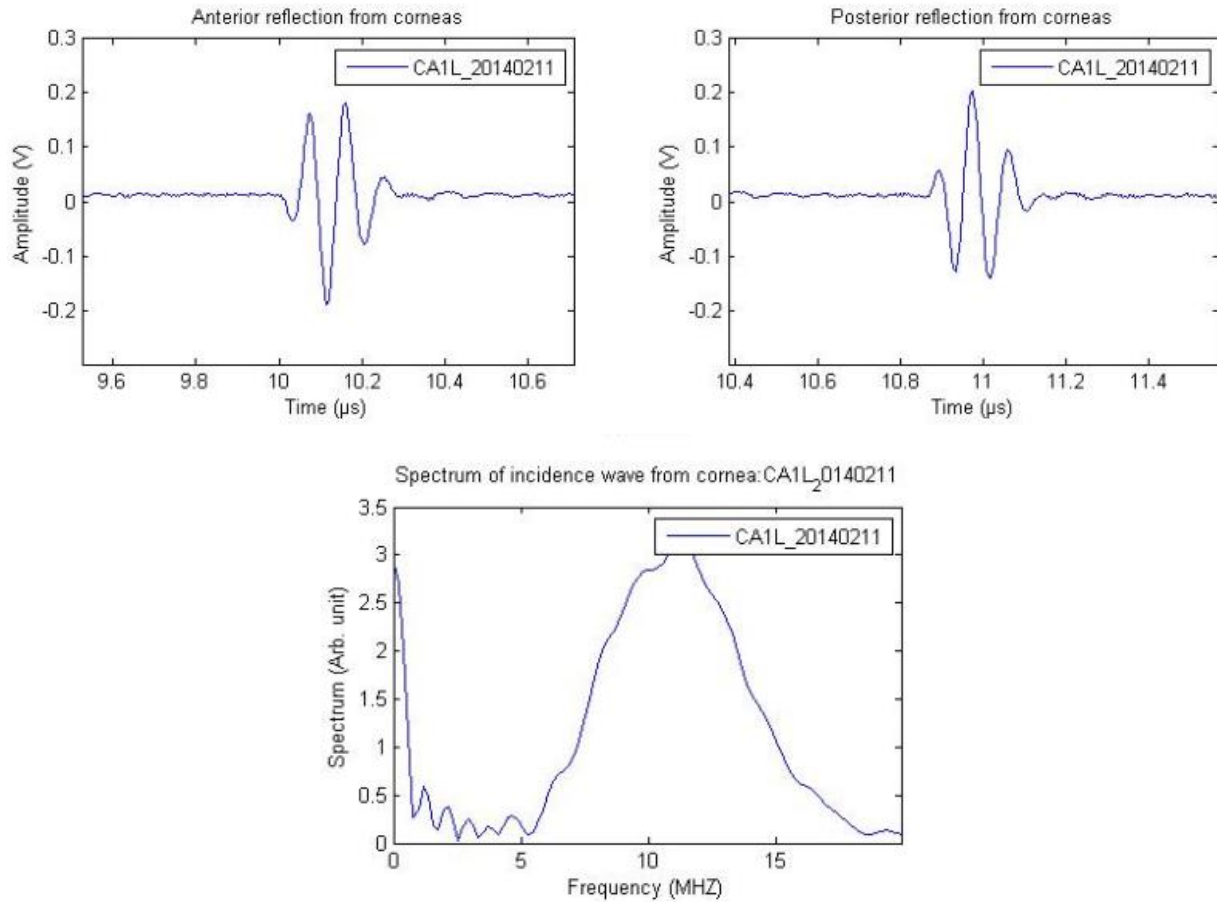


Figure 2-6: Sample acoustic impedance calculation output

Mechanical testing data was processed in MATLAB. A MATLAB script that processed the data files was developed by Dr. Hugh Morris. The program would require the input of the sample cross-section dimensions, thickness and width previously gathered from a pachymeter and calipers, respectively. The program would plot and fit the individual stress-strain curves and compile all the curves on the same plot. A sample output of the curves can be viewed below in Figure 2-7. The program would obtain the secant modulus for each sample at strains of 1%, 2%, 3%, 4%, and 5%. Additionally, the program would obtain additional fitting parameters and other moduli that can be used for further analysis.

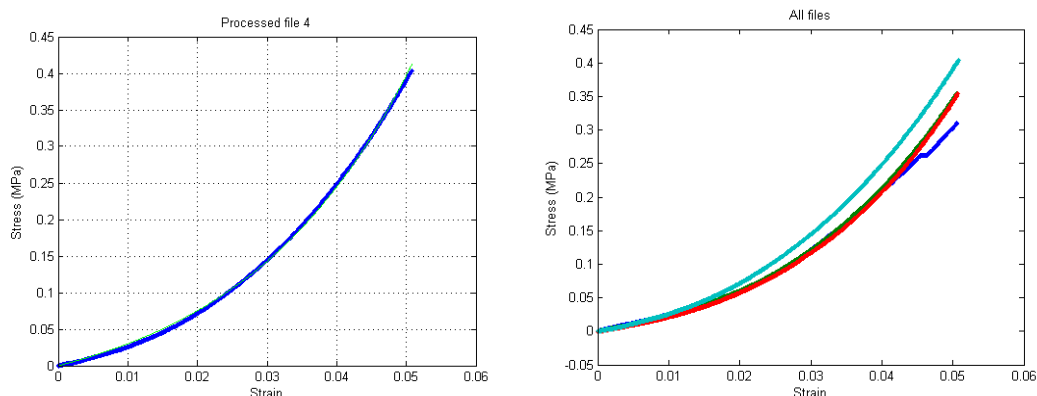


Figure 2-7: Sample RSA output

After the completion of the collagen assay and spectrophotometry, absorbance values for standard solutions and samples were exported into Microsoft Excel software. All calculations for the collagen assay were performed in Microsoft Excel software. Standard curves were created and were given a linear fit to obtain a slope and intercept. This was used to calculate sample concentration as viewed in Equation 1 below. The concentration was then utilized with sample dilution volume to obtain sample weight of collagen as seen in Equation 2 below. The weight of the collagen was then divided by sample dry weight to obtain a percentage of collagen content per dry weight as seen in Equation 3 below.

Equations 2 and 3 were manipulated to produce Equations 4 and 5 below to determine the amount of tissue needed to be within the sensitivity of the collagen assay. Ideally, collagen percentage would be around 5 mg/ml, the central value of the standard solutions. Collagen content was assumed to be 70% of dry tissue dry weight. This suggested that approximately 20 mg of dry tissue should be used for the assay or 100 mg of wet tissue (assuming 80% of wet tissue is water).

Collagen Assay % Calculation

- (1) $\frac{\text{Sample absorbance} + \text{intercept}}{\text{slope}} = \text{Sample concentration} \left(\frac{\mu\text{g}}{\text{ul}} \text{ or } \frac{\text{mg}}{\text{ml}} \right)$
- (2) $\text{Sample concentration} \left(\frac{\text{mg}}{\text{ml}} \right) * \text{dilution volume (ml)} = \text{Weight of collagen (mg)}$
- (3) $\frac{\text{Weight of collagen}}{\text{Dry weight of tissue}} * 100 = \text{Collagen \% of dry weight}$

Tissue Sample Weight for Collagen Assay Calculation

- (4) $\text{Sample concentration} \left(\frac{\text{mg}}{\text{ml}} \right) * \text{dilution volume (ml)} = 5.0 \frac{\text{mg}}{\text{ml}} * 3 \text{ ml} = 15.0 \text{ mg collagen}$
- (5) $\text{Dry weight of tissue} = \frac{\text{Weight of collagen}}{\text{Collagen \% of dry weight}} * 100 = \frac{15.0 \text{ mg collagen}}{.70} = 21.4 \text{ mg dry weight}$

Chapter 3: Results

For the results, acoustic impedance will be abbreviated as AI, pepsin-soluble collagen as SC, pepsin-insoluble collagen as ISC, absorbance as Abs. Samples are grouped based on when testing was performed. Samples 1 and 2 are the first group, and samples 3 through 6 are the second group. Samples were tested in numerical order. OS and OD indicate a matched pair.

Tables 3-1 and 3-2 below were compiled from all the processed data from the acoustic, mechanical, and collagen testing and analyses. All eyes underwent acoustic and mechanical testing, as seen in Table 3-1. Samples 4, 5, and 6 went through the collagen assay, which can be viewed alongside acoustic data in Table 3-2.

Table 3-1: Tabulated acoustic and mechanic data

Sample Number	AI (MRayl)	Modulus at 3% strain (MPa)	Modulus at 5% strain (MPa)
OS1	1.63	4.22	6.29
OD1	1.69	3.96	6.77
OS2	1.63	3.87	6.81
OD2	1.67	4.96	8.02
OS3	1.69	5.32	6.41
OD3	1.69	2.17	5.23
OS4	1.66	5.10	9.26
OD4	1.63	2.18	5.41
OS5	1.67	0.90	2.81
OD5	1.66	3.42	7.89
OS6	1.74	0.90	2.49
OD6	1.67	2.82	7.22

Table 3-2: Tabulated acoustic, mechanic, and collagen data

Sample Number	AI (MRayl)	ISC (mg)	Total collagen (mg)	Dry Weight (mg)	ISC collagen (mg/mg)	Total collagen (mg/mg)
OS4	1.66	25.49	25.91	31.80	0.80	0.81
OD4	1.63	-16.87	-9.71	22.00	-0.77	-0.44
OS5	1.67	21.84	23.99	24.60	0.89	0.98
OD5	1.66	40.58	41.53	30.40	1.33	1.37
OS6	1.74	18.31	20.08	28.80	0.64	0.70
OD6	1.67	24.12	30.81	23.50	1.03	1.31

Figures 3-1 and 3-2 are the acoustic plots produced by the first and second groups, respectively. For each figure, the upper left contains the anterior reflections, the upper right is posterior reflections, and the lower portion is the wave spectrum of the incident wave.

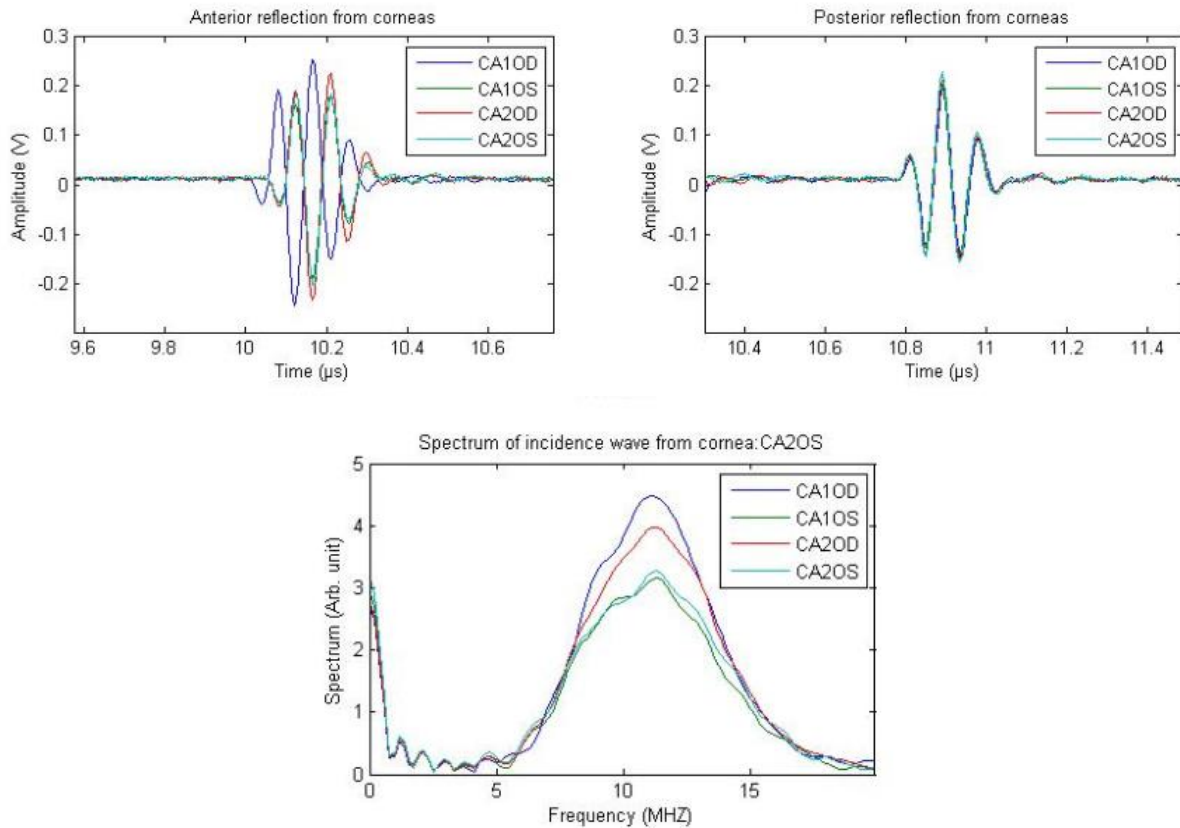


Figure 3-1: Acoustic data for samples 1 and 2; (upper left) anterior reflection, (upper right) posterior reflection, (lower) wave spectrum

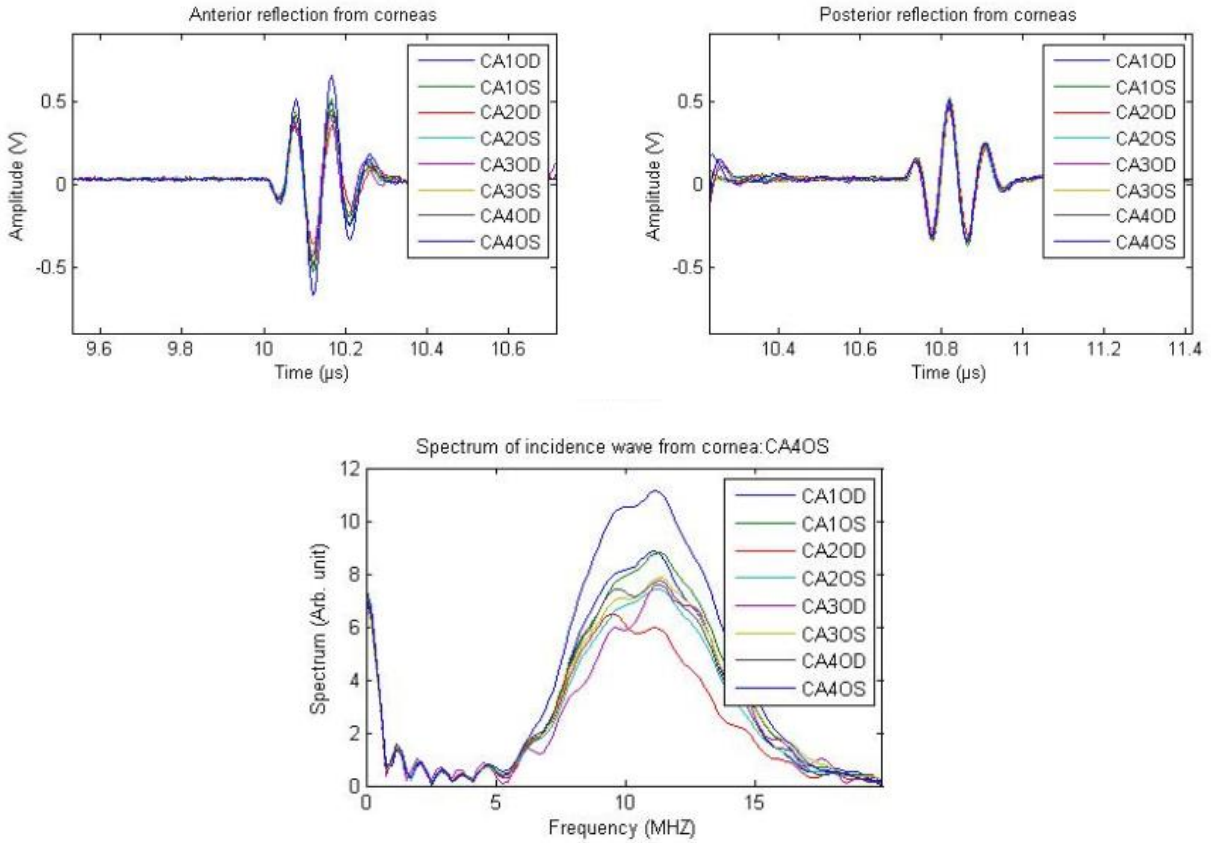


Figure 3-2: Acoustic data for samples 3 through 6; (upper left) anterior reflection, (upper right) posterior reflection, (lower) wave spectrum

Figures 3-3 and 3-4 below are the compiled stress-strain curves for the first and second group, respectively. These curves were used to obtain the Young's secant modulus at each strain level.

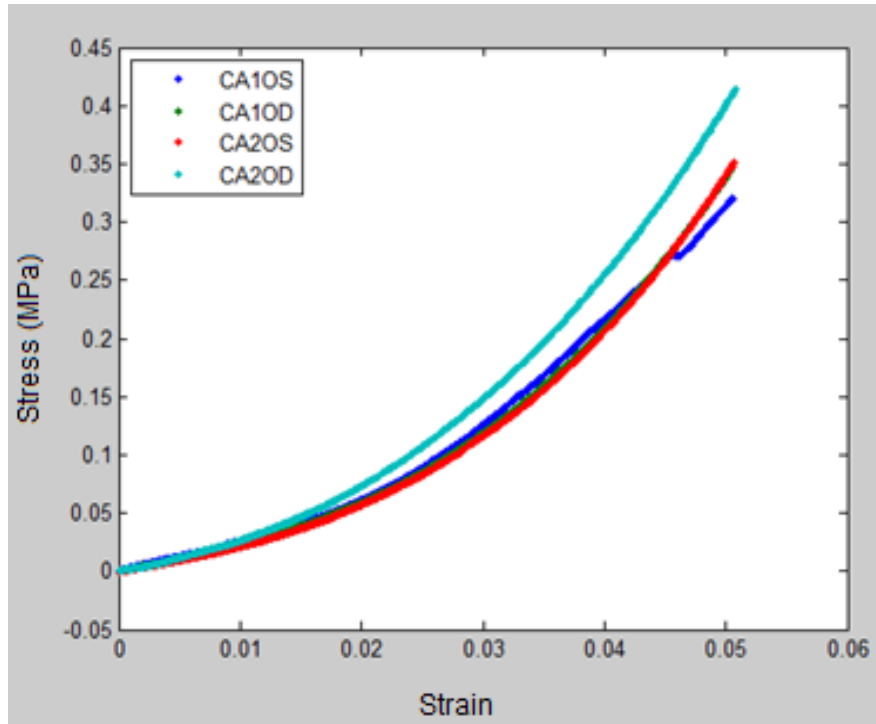


Figure 3-3: Stress-strain curves for samples 1 and 2

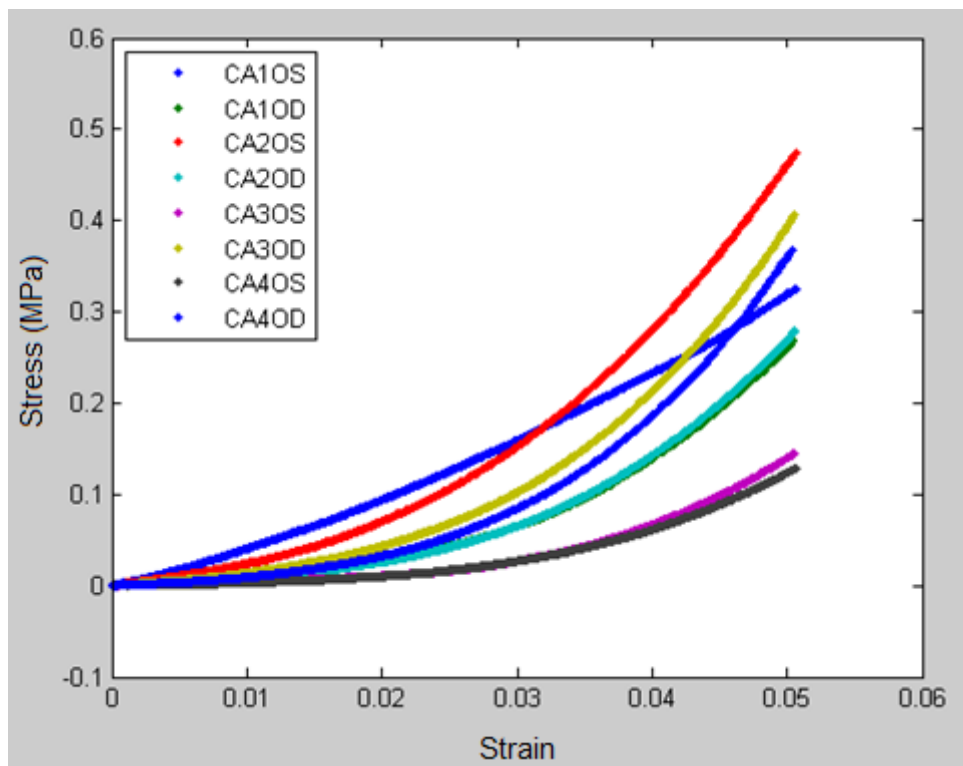


Figure 3-4: Stress-strain curves for samples 3 through 6

Figure 3-5 below is a photo of the cuvettes after the production of a chromophore in the collagen assay. The cuvettes follow the key in Table 3-3. The first row is soluble collagen, and the second row is insoluble collagen. The bottom two rows are the hydroxyproline standard solutions used to create the standard curve.



Figure 3-5: Standard solutions and sample solutions in cuvettes at the completion of the collagen assay reactions

Table 3-3: Key for cuvettes in Figure 3-5 above

CA4OS SC	CA4OD SC	CA5OS SC	CA5OD SC	CA6OS SC	CA6OD SC
CA4OS ISC	CA4OD ISC	CA5OS ISC	CA5OD ISC	CA6OS ISC	CA6OD ISC
0.0 ug Hyp	0.2 ug	0.4 ug	0.8 ug	1.0 ug	
1.5 ug	2.5 ug	5.0 ug	7.0 ug	9.0 ug	

Figure 3-6 below contains the standard curve of known hydroxyproline concentrations at an absorbance of 555 nm. This curve was used to determine sample solution concentrations for samples 4, 5, and 6. Table 3-4 contains data from the collagen assay and values calculated using Equations 1, 2, and 3. SC used a 1 ml dilution, and ISC used a 3 ml dilution. The last column of Table 3-3 expresses the type of collagen as a percentage of the dry weight.

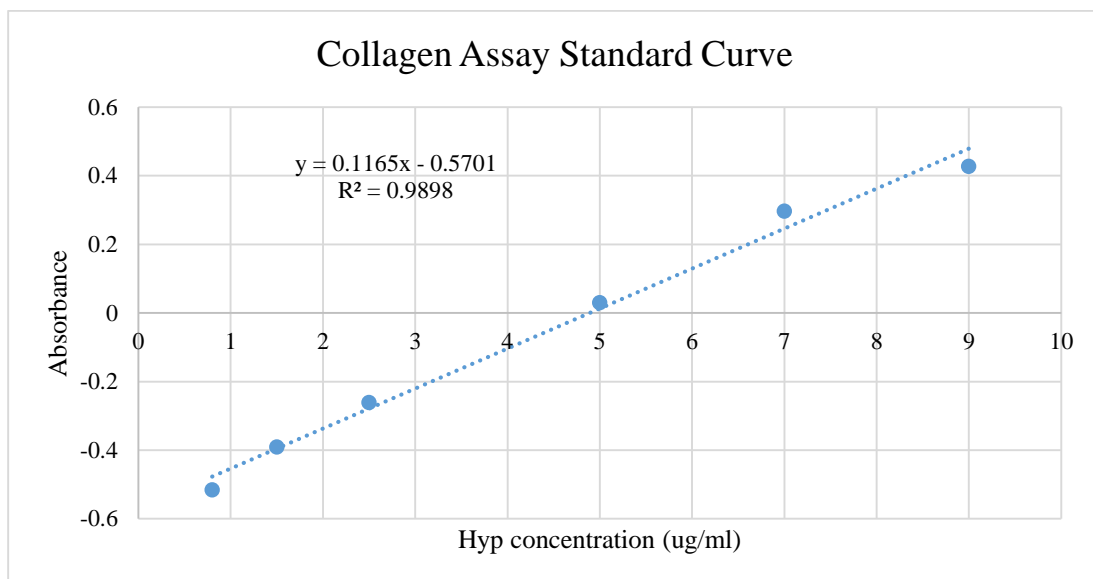


Figure 3-6: Hydroxyproline standard curve at 555 nm absorbance

Table 3-4: Collagen assay values

Sample	Dry Weight (mg)	Abs	Ref. Soln (mg/ml)	Dry weight (mg)	Collagen/ Dry Wt (mg/mg)	Collagen/ Dry Wt (%)
1OS SC	31.8	-0.52	0.42	0.42	0.01	1.32
1OD SC	22.0	0.26	7.16	7.16	0.33	32.56
2OS SC	24.6	-0.32	2.14	2.14	0.09	8.71
2OD SC	30.4	-0.46	0.95	0.95	0.03	3.12
3OS SC	28.8	-0.36	1.77	1.77	0.06	6.15
3OD SC	23.5	0.21	6.68	6.68	0.28	28.44
4OS ISC	31.8	0.42	8.50	25.49	0.80	80.15
4OD ISC	22.0	-1.23	-5.62	-16.87	-0.77	-76.69
5OS ISC	24.6	0.28	7.28	21.84	0.89	88.79
5OD ISC	30.4	1.01	13.53	40.58	1.33	133.48
6OS ISC	28.8	0.14	6.10	18.31	0.64	63.57
6OD ISC	23.5	0.37	8.04	24.12	1.03	102.64

Figure 3-7 below contains the data from the study conducted by He and Liu. This data will be used for comparison purposes.

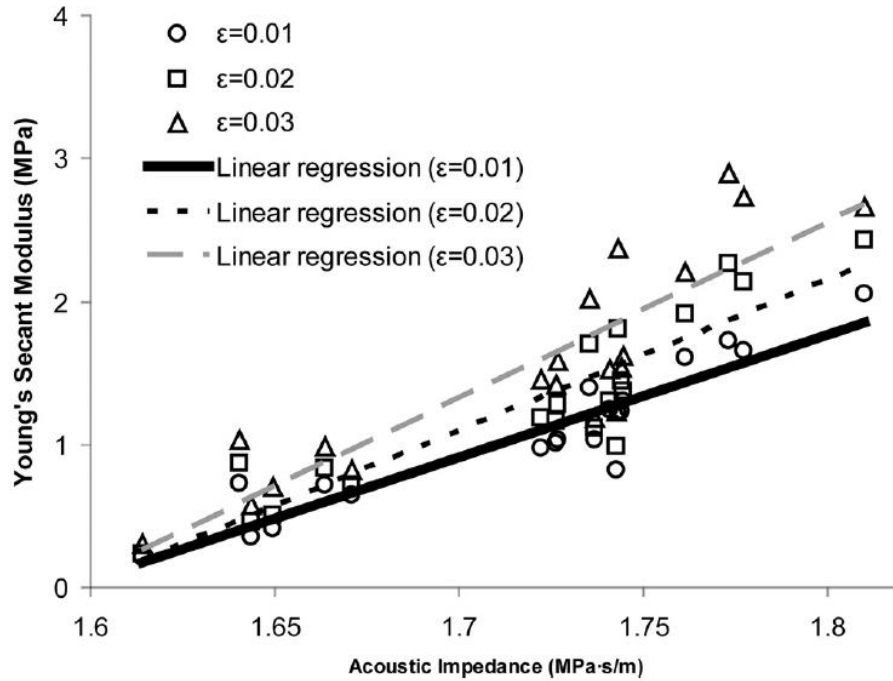


Figure 3-7: Historical acoustic vs mechanics data [6]

Figures 3-8, 3-9, and 3-10 were created in Microsoft Excel from Tables 3-1 and 3-2.

Figures 3-8, 3-9, and 3-10 plot mechanical testing, insoluble collagen content, and total collagen content as a function of acoustic impedance, respectively. Total collagen content was obtained from adding soluble and insoluble collagen contents.

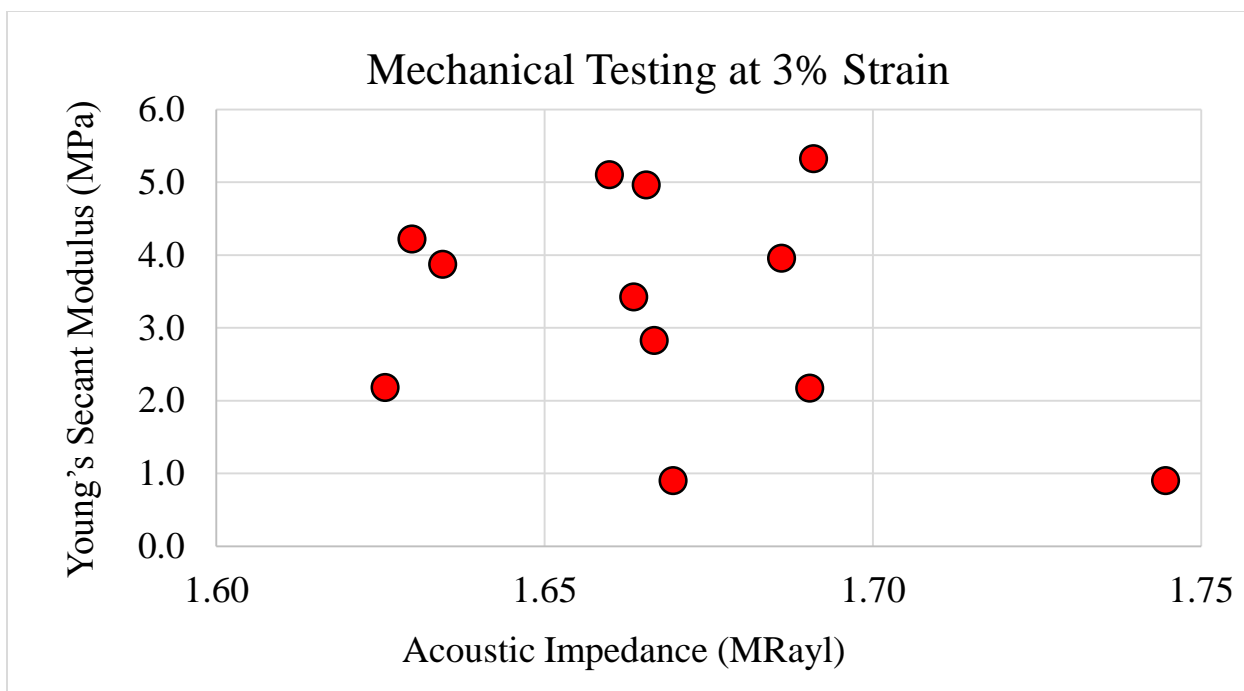


Figure 3-8: Acoustic vs Mechanical data

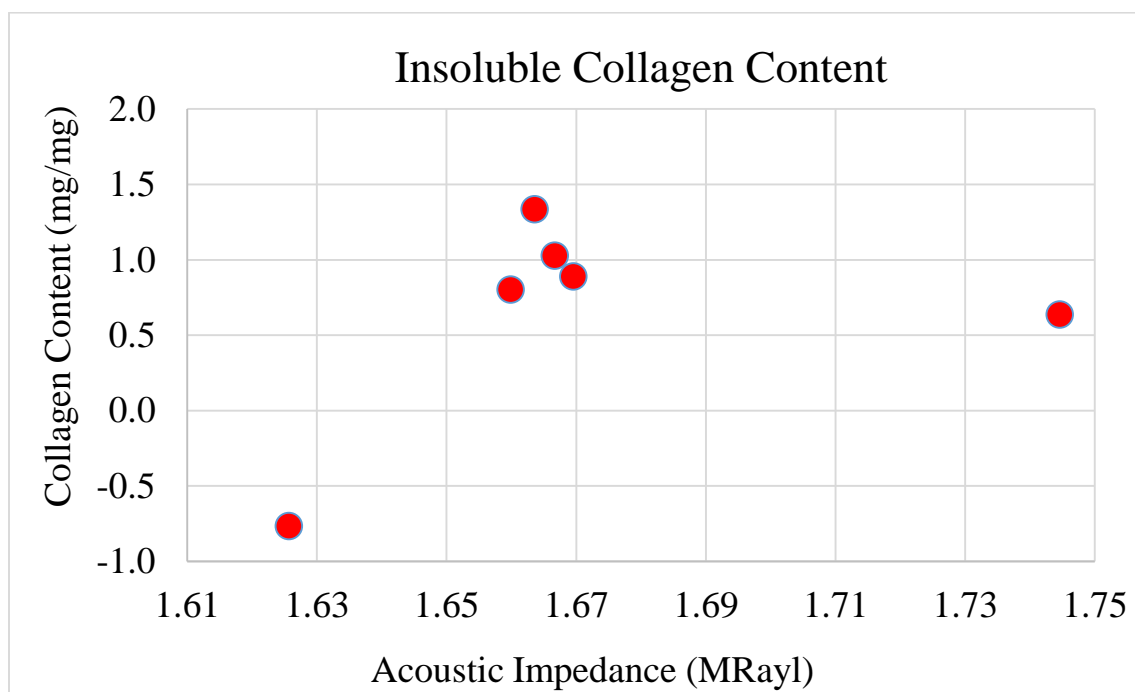


Figure 3-9: Acoustic vs insoluble collagen content

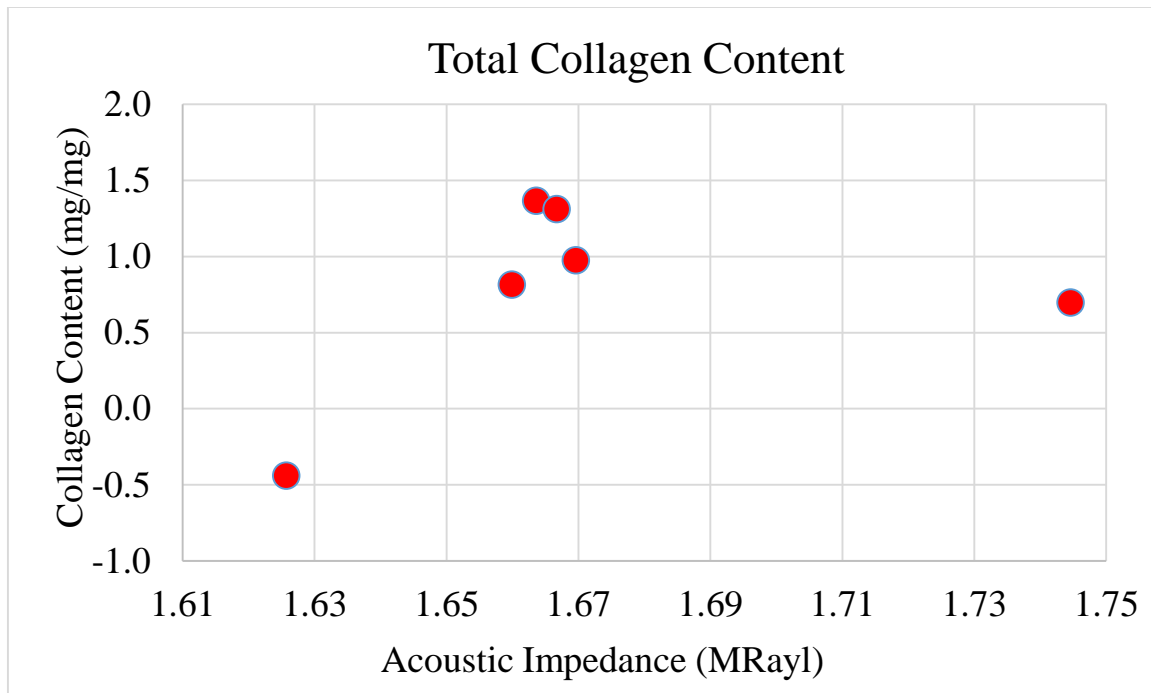


Figure 3-10: Acoustic vs total collagen content

Due to a low sample size (N=12), statistics were not performed for this study.

Chapter 4: Discussion

The previous study conducted by He and Liu was used as a reference to evaluate the accuracy/inaccuracy of the current data set, specifically by comparing the acoustic and mechanical data between Figures 3-7 and 3-8. The range of acoustic values is between 1.60 and 1.75 MRayl for the current study, and the range of acoustic values is between 1.60 and 1.80 MRayl for the previous study. This suggests that the acoustic measurements and procedure for this study may be appropriate. The upper limit of the mechanical values for this study is 6.0 MPa, and the upper limit for the previous study is approximately 3.0 MPa. For the current study, over 50% of the Young's secant moduli at 3% strain are above 3.0 MPa, suggesting potential inaccuracies in the mechanical testing and measurements likely due to imperfect alignment of samples. Additionally, Figure 3-1 and 3-2 indicate consistent wave propagation. To note, in Figure 3-1, the waves do not perfectly overlap, as the distance between the samples and the ultrasound transducer are manually controlled, shifting the time location of the wave. This is accounted for in the data processing.

Although sample size was very limited in the present study and could be responsible for not reproducing the correlation seen in He and Liu's study, issues with the mechanical testing may offer additional explanation. Ideally, mechanical testing would be conducted within 3 hours post mortem. As many samples were tested during the same period of time, some samples were tested 5-6 hours post mortem. Tissue swelling and drying may have had a significant effect in this time. Specifically in Figure 3-4, lower curves (CA3OS and CA4OS) typically indicate weaker tissue. Both CA3OS and CA4OS were tested at later times which could have experienced significant swelling. Tissue swelling can be a result of the transport medium, which slows, but does not completely stop, corneal swelling. In Figure 3-4, CA1OS has a relatively higher linear

appearance, and this may be a result of the tissue being dry due to a lack of medium application. Other transport mediums can be considered in the future, such as mineral oil, and sample testing can be limited to one pair per testing period to reduce post mortem testing time. Additionally, clamping and alignment may have significant effects on the mechanical testing. In Figure 3-3, CAIOS suggests that the tissue slipped from the clamps during the last portion of the ramp procedure. Poor alignment may have less predictable results on the curves, but may also offer an explanation for the wide range of curves seen in Figure 3-4. Samples were also clamped over the sclera, leaving the limbus within the testing region. A study has suggested that the distribution of collagen fibers in the limbus differs from the central cornea [14]. This may have an unpredictable effect on the mechanical testing, as the acoustic testing may only correlate with the mechanical properties of the central cornea.

There are many strengths and weaknesses for collagen assay that were discovered while developing the assay. The assay is relatively cheap when compared to many commercial assays currently available, and most of the resources and equipment are readily available at most research institutions. The assay offers the ability to expand a solubility profile to further characterize collagen of the cornea, including additional acid and salt digests [7]. The assay does offer potential correlations with total collagen content and insoluble collagen content, as seen in Figure 3-9 and 3-10. The negative concentrations were clearly an error in the measurement which was likely due to the calibration issues of the spectrophotometer. As this procedure was adapted from sclera and tendon, the sample mass and reagent volumes may need to be adjusted for the cornea. The protocol required pipetting very small volumes ($<10\ \mu\text{l}$). These volumes were very difficult to visually assess, and may have contributed errors at many points in the procedure. Future iterations of the collagen assay will utilize larger volumes and use serial dilutions to

improve volume and concentration accuracy. Homogenization was very difficult, as the cornea would not become finely mixed in solution, frequently sticking to the homogenizer blades. This may be a result of the immersion medium (i.e., Optisol) that was not fully removed from the tissue. In future experiments, the tissue will be allowed to soak in distilled water to remove transport medium, to hopefully improve homogenization.

As seen in Table 3-2, collagen contents are scattered, as some results were above 100% dry weight and one was below 0%. Another study suggests that 70% of total dry mass of the cornea is collagen [15]. As seen in Table 3-4, pepsin-soluble collagen (SC) contents were very low when compared to pepsin-insoluble collagen (ISC). A study of the sclera collagen content demonstrated similar orders of magnitude of SC and ISC in sclera [7]. These values can be compared against other literatures values to validate the collagen assay for the cornea. The spectrometer used in this study may also require calibration. A visual assessment of the hydroxyproline standard solutions in Figure 3-5 shows a gradient of colors, from yellow to orange to red, with the exception of one or two standards. Many of the sample solutions visually fall within this gradient, but the absorbance values do not reflect the visual assessment. A plate reader and reflectance values may be used in future assessments of the solutions.

Chapter 5: Conclusion

Collagen content may offer an explanation for the correlation between acoustic and biomechanical properties of the cornea in canine models. The initial set of data is limited but demonstrates a potential correlation with the acoustic impedance with regards to pepsin-soluble collagen and total collagen content. The hydroxyproline assay developed for the cornea has undergone preliminary testing and requires much refining to the protocol.

For future work, the protocol can be validated and expedited by analyzing only total collagen content and comparing those values against accepted literature values. Collagen crosslinking can be directly investigated in future studies, potentially through fluorescent labeling, and can be compared against insoluble collagen values from the assay. The collagen assay can also be applied to additional studies, in which acoustic and mechanics of the cornea samples have already been characterized, and the collagen content may offer further insights.

There is a clear need for a noninvasive device that can obtain accurate *in vivo* measurements of the mechanical properties of the eye. Visual impairments result from the changing of the eye's microstructure and its associated mechanical properties and require the constant use of lenses or surgical intervention. Understanding the mechanical and structural properties on both the micro- and macro-scale of the eye can lead to improved preventative treatment of vision deterioration.

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Appendix: Protocols

Tensile Testing (RSA) Protocol

Prepared by Marty Spang on 1/22/2014

Location: Koffolt 432

- Turn on machine, back left-hand corner
- Start TA Orchestrator
- Prepare the strip
 - cut and measure width at 3 different spots, then average
- Put clamps into setup
 - long bottom, short top
- Clamp top of cornea, limbus barely visible, straight
 - leave extra sclera when removing corneas from globes
- Apply Optisol every 1-2 minutes
- Control>Gap control panel
 - turn on motor
- Control>Edit/start test
- ***Name File***
 - CA001D_Cor-NT_DMA_1Hz16cylce_pre2p0g_20140121
 - D is right, S is left
- Edit geometry: Enter width & thickness, length doesn't matter
- Check "Read test fixture gap"
- Test Setup>Stored test setups
 - Liu_DMA
 - Edit Test>Wave>Send Wave
 - ***Check settings***
 - Check all 4 regions are 4 seconds with " $0.0015 \cdot \sin(6.283 \cdot t)$ " 350 points Per Zone
- Control>Gap control

- Start at 0 g
 - Up to 4-6 g and down to 0 g
 - Repeat 5 times
- Run DMA test at around 2.0 g
- Bring to 0.5 g through Gap control
- Then Export to .TXT file
- Wait total of 5 minutes at 0.5 g
- ***Tighten screws***
- ***Name File***
 - CA001D_Cor-NT_Ramp_0p1_pre0p5g_20140121
- Test Setup>Stored test setups
 - Liu_Ramp
 - ***Check settings***
 - 2 regions, 25 => 5% strain, 350 points per zone, 0.001 extension value, Hencky extension mode
- Run test
- Export test to .TXT file
- Save files to portable flash drive
- Remove tissue, clean up

Hydroxyproline (Hyp) assay protocol

Adapted by Martin Spang on 4/1/2014

Based on protocols prepared by Joel Palko & Jeff Tonniges

Materials & Reagents:

- 1.7 ml centrifuge tubes
- 1.7 ml cryo tubes (or other tubes that can be autoclaved)
- Spectrophotometer cuvettes/plate
- NaOH pellets
- Distilled water (DW)
- L-hydroxyproline powder
- Chloramine-T powder
- 2-propanol (isopropyl alcohol)
- Perchloric acid
- p-dimethylaminobenzaldehyde
- Sodium acetate trihydrate
- Citric acid

Reagent Preparation:

Acetic Acid (0.5 M)

1. Mix 25 ml 1.0 N acetic acid and 25 ml DW

Acetate-citrate buffer (pH 6.5)

1. Can be stored up to 12 months, wrap in foil, keep away from light
2. Dissolve 120 g sodium acetate trihydrate, 46 g citric acid, 24 gram NaOH in 8 ml DW
3. Titrate to pH of 6.5 with acetic acid, ~ 4 ml

Sodium Hydroxide (10 N)

1. Dissolve 600 mg of NaOH into 1.5 ml DW

Hydroxyproline standard solutions:

1. Dissolve 1 mg L-hydroxyproline into 1 ml DW for stock solution of 1 mg/ml (1 ug/ul) hydroxyproline
2. Use serial dilutions of stock solution to make standard samples

Chloramine T reagent (0.056 M)

1. Dissolve 127 mg of Chloramine T in 2 ml of 50% 2-propanol
2. Bring total volume to 10 ml with acetate-citrate buffer
3. Use a facemask, make under fume hood

Ehrlich's reagent

1. Mix 6.67 ml 2-propanol with 3.33 ml perchloric acid
2. Dissolve 1.5 g of p-dimethylaminobenzaldehyde in the propanol-perchloric acid solution
3. Use a facemask, make under fume hood

Hydroxyproline assay (continued)

Directions:

1. Lyophilize and weigh dry tissue
2. Place sample into 1.7 ml centrifuge tubes, add 1 ml of 0.5 M acetic acid with 0.1 mg/ml pepsin
3. Shake for 24 hours at 4°C
4. Centrifuge at 15000 rpm for 15 minutes, transfer supernatant of pepsin-soluble collagen
5. Homogenize remaining tissue in 3 ml DW
6. Prepare hydroxyproline standards => 0.0, 0.2, 0.4, 0.8, 1.0, 1.5, 2.5, 5.0, 7.0, 9.0 ug Hyp/10 ul DW
7. Add 20 ul 10 M NaOH soln, add 20 ul DW
8. Autoclave samples for 30 min at 121°C => wait 10-15 min for pressure to lower => let samples cool
 - a. During autoclaving samples, prepare chloramine-T reagent solution
9. Add 450 ul chloramine T reagent (0.056 M) => incubate at RT for 25 min
 - a. During incubation, prepare Ehrlich's reagent solution
10. Add 500 ul Ehrlich's reagent => incubate at 65°C for 20 min
11. Measure absorbance at 555 nm using spectrophotometer => collagen content obtained from standard curve using pure hydroxy-proline, assume 14% Hyp in collagen for calculation